

# Noninvasive detection of filaggrin gene mutations using Raman spectroscopy

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**Abstract:** Knowledge of the existence of filaggrin (FLG) gene mutations might be helpful for a subclassification of patients with atopic dermatitis (AD) which can be used to introduce individualized treatments. In this work the filaggrin content in the skin is assessed using Raman spectroscopy and the results are compared to FLG genotyping of Mexican-mestizo patients. Results showed that the 2282del4 and R501X mutations present in the European population but absent in people of Asian or African descent are also present in the Mexican-mestizo population. The results also showed that patients with filaggrin gene mutations presented lower filaggrin concentrations measured using the vector correlation of their skin Raman spectra and a fixed spectrum of pure human recombinant filaggrin, these results indicate that Raman spectroscopy may be used as a noninvasive tool to detect FLG gene mutations.

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## 1. Introduction

Filaggrin (FLG) is a key protein required for the formation of the stratum corneum (SC) barrier [1]. The importance of filaggrin gene mutations as a predisposing factor for atopic dermatitis (AD) has modified the concept of AD, which is now considered a disease based on a primary genetic epithelial barrier defect with consecutive, secondary modified immune responses [2].

FLG haplo-insufficiency contributes to the impairment of the skin barrier in AD and increases the risk for severe courses of AD and concomitant development of asthma as well as the development of sensitizations to allergens and haptens such as nickel via the cutaneous route [2].

Knowledge of the existence of FLG gene mutations might be helpful for a subclassification of patients with AD which can be used to introduce individualized treatments [2], however routine genotyping of multiple but rare FLG gene mutations might be difficult in daily clinical practice therefore several attempts have been made to detect FLG gene mutations noninvasively [1,3,4].

Kezic et al. [1] used confocal Raman microspectroscopy to measure natural moisturizing factor (NMF) as a function of SC depth, the results suggested that these measurements could be used as a marker of FLG status, similar results were obtained by O'Regan et al. [3] also measuring NMF, it is worth noting that these attempts did not measure filaggrin content directly but inferred the lack of filaggrin when low NMF quantities were measured.

In a previous work [4] skin filaggrin content was estimated directly and noninvasively on newborn infants using Raman spectroscopy and principal component analysis (PCA), these infants were monitored during a year and the ones with lower filaggrin content as determined using Raman spectroscopy later developed atopic dermatitis, however no genotyping was performed on these subjects.

In this work the filaggrin content in the skin is assessed using Raman spectroscopy and the results are compared to their FLG genotype in order to validate the use of Raman spectroscopy as a noninvasive tool to detect FLG mutations.

## 2. Method

In this study 19 Mexican-mestizo patients with major complains of pruritus and dry skin were analyzed, informed consent was obtained from all participants and the study was approved by the ethics committee of the Hospital 'Dr Ignacio Morones Prieto'.

Raman scattering measurements were performed on the inner area of the forearm of the 19 patients, approximately 10 cm above the wrist. The measurements were made at room temperature using a Raman Systems R3000 spectrometer (Ocean Optics, Dunedin, FL, USA) with a 785 nm laser diode, a spectral resolution of  $8 \text{ cm}^{-1}$  and a laser power of 90 mW. The irradiance of this laser diode is below the ANSI standard for skin and none of the participants showed any kind of discomfort when the measurement was performed. The measurements were made in the 200 to  $1800 \text{ cm}^{-1}$  spectral range and the instrument was calibrated using a Teflon standard before each round of measurements.

All the measured spectra were preprocessed by subtracting a fifth grade polynomial to the raw spectra applying the fluorescence removal algorithm proposed by Zhao et al. [5], also known as the Vancouver algorithm, in order to remove the background NIR fluorescence and leave the pure Raman signal. Figure 1(a) shows a raw Raman spectrum of the skin, and Fig. 1(b) shows a Raman spectrum of the skin with the fluorescence background subtracted.

DNA was obtained from buccal cells using the Gentra Puregen (Qiagen®) protocol using a glycogen and isopropanol precipitation with freezing for higher recovery. The filaggrin gene was amplified by polymerase chain reaction (PCR) based on sequences published by Smith et al. [6]. For the R501X mutation detection a 311pb PCR fragment was incubated with 5 units of Nla III restriction enzyme (New England Biolabs®) and the products were resolved by a 3% agarose 1000 (Invitrogen®) or 12% acrylamide gel electrophoresis; normal sequence

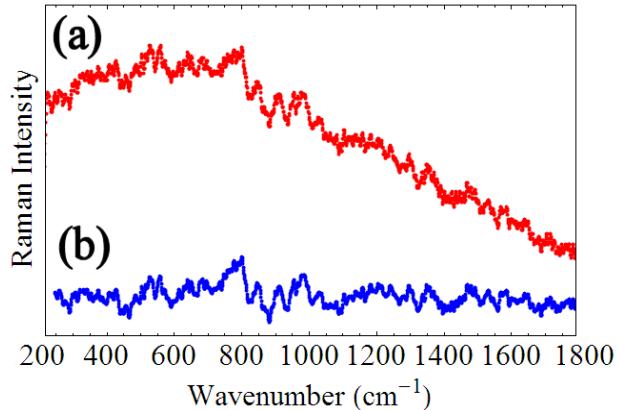


Fig. 1. (a) Raw Raman spectrum of skin, (b) Raman spectrum of skin with the background fluorescence signal subtracted.

produces a 204 and 107 pb fragments and the mutant sequence generates the same 107 pb fragment plus additional 128 and 76 pb fragments. The 2284del4 mutation which creates a new restriction site was detected incubating an 811pb filaggrin DNA fragment with the Dra III (New England Biolabs®) enzyme; products were resolved on a 2% regular agarose gel.

The Raman spectrum of pure filaggrin human recombinant protein (GenWay Biotech, Inc., San Diego, CA) was measured in order to have a fixed spectrum that will help analyze the Raman measurements and assess their FLG concentration. Figure 2 shows the measured Raman spectrum of the pure filaggrin protein.

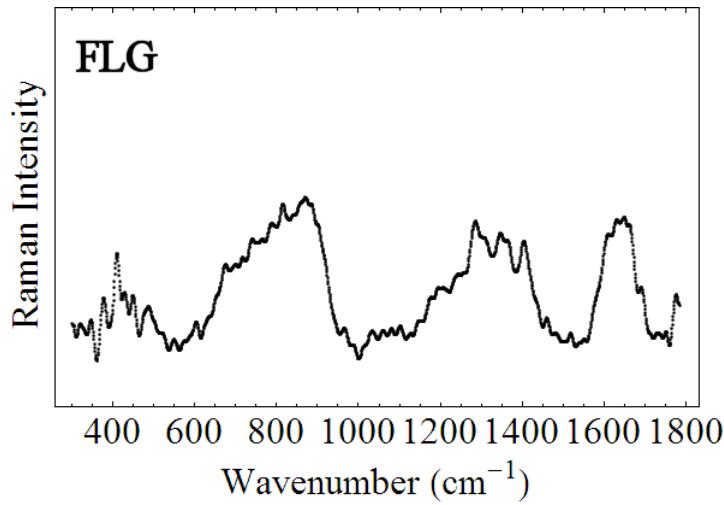


Fig. 2. Raman spectrum of pure human recombinant filaggrin.

The amount of FLG on the measured samples was estimated by performing a vector correlation between the pure FLG spectrum (Fig. 2) and the spectra obtained from the patients (Fig. 1(b)), this vector correlation was performed using the Mathematica™ program (Wolfram Research, Inc., Champaign, Illinois). This approach gives an advantage over principal component analysis since it does not need a large data sample for good accuracy and can be performed over just one spectrum.

### 3. Results

The genetic analysis showed that 8 out of the 19 patients (42%) presented a FLG mutation. These 8 patients presented the 2282del4 FLG mutation, 2 of which (10.5%) where

homozygous and 6 (31.5%) heterozygous, whereas 1 (5.2%) resulted in a compound heterozygote for the 2282del4 and the R501X mutations.

These genetic results were compared to the filaggrin content estimation, a lower vector correlation value of the spectra with the pure filaggrin spectrum would indicate a lower filaggrin concentration relative to the rest of the vector correlation results.

Figure 3 shows the results of the vector correlation for the patients with a filaggrin mutation (FLG -) and without a filaggrin mutation (FLG +). The patients with a filaggrin mutation presented an average vector correlation of 0.286 (SD = 0.082) while the patients without a filaggrin mutation showed an average vector correlation of 0.4 (SD = 0.08).

From Fig. 3 it can be seen that 7 out of 8 patients with a filaggrin mutation (87.5%) presented a correlation lower than 0.35 and 8 out of the 11 patients without the R501X and 2284del4 filaggrin mutations (72.7%) presented a correlation higher than 0.35 (dotted line in Fig. 3).

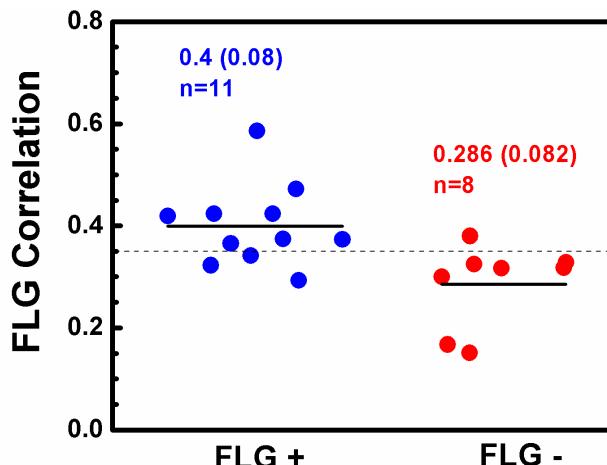


Fig. 3. Vector correlation of the Raman spectrum of pure human filaggrin with the Raman spectra of the skin of patients with pruritus and dry skin, the FLG + points show the patients without a FLG mutation, while the FLG - points represent patients with a FLG mutation.

#### 4. Conclusions

The performed genotyping showed that the 2282del4 and R501X mutations present in the European population [7] but absent in people of Asian or African descent [8] are also present in the Mexican-mestizo population.

These findings could help to determine other filaggrin mutations in other Latin-American populations.

Even though the patients that participated in this study did not undergo the diagnostic criteria for atopic dermatitis, the percentage that presented FLG mutations (42%) is not too far from the percentage of FLG mutations present in the Caucasian population, where loss-of-function mutations are detectable in about one-third of patients with AD [2], a study with a larger number of Mexican-mestizo patients who comply with the diagnostic criteria for AD is currently underway in order to compare the incidence of these mutations to the European population.

The Raman measurements showed that patients with filaggrin gene mutations presented lower filaggrin concentrations measured using the vector correlation of their skin Raman spectra and a fixed spectrum of pure human recombinant filaggrin, these results indicate that the cross-correlation of the filaggrin Raman spectrum with the Raman spectra of skin can be an indicator of filaggrin gene mutations.